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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 11 June 2002 with an application for Letters Patent number 519504 made by AUCKLAND UNISERVICES LIMITED.

Dated 12 June 2003.

Neville Harris
Commissioner of Patents

Patents Form No. 4 Our Ref: TJ503974

Patents Act 1953

PROVISIONAL SPECIFICATION

MEASUREMENT OF MELANOCORTIN PEPTIDES AND USES THEREOF

We, **AUCKLAND UNISERVICES LIMITED**, a New Zealand company, of Level 10, 70 Symonds Street, AUCKLAND New Zealand do hereby declare this invention to be described in the following statement:

PT0411732

MEASUREMENT OF MELANOCORTIN PEPTIDES AND USES THEREOF

TECHNICAL FIELD

The present invention relates to methods for the detection and/or quantitation of melanocortin peptides and their use for risk assessment and diagnosis of disease.

BACKGROUND

Obesity and type 2 diabetes are major health problems worldwide and are a major threat to health and well-being. Over the last few years significant advances have been made with respect to the molecular determinants of energy balance and insulin resistance. Critical elements of this control system are hormones secreted in proportion to body fat, including leptin and insulin, and their central nervous system targets such as neuropeptide Y and the hypothalamic melanocortin system. Recently proopiomelanocortin and MC4-R have been identified as key targets mediating leptin's activities in the hypothalamus.

Pro-opiomelanocortin (POMC), produced in the pituitary and brain and to a lesser extent in numerous peripheral tissues, is the large precursor protein from which melanocortin peptides α-melanocyte stimulating hormone (MSH) and adrenocortiocotropin (ACTH) and fragments thereof, are derived. The products of POMC undergo a series of complex, tissue specific processing events such as further proteolytic cleavages, phosphorylation, α-amidation and NH_2-terminal acetylation which influence their biological activities. $ACTH_{1-13}NH_2$ exists as α-MSH and desacetyl-α-MSH. α-MSH, which is acetylated at the N-terminus and amidated at the COOH terminus, is a post translationally modified derivative of $ACTH_{1-13}$ NH_2 (desacetyl-α-MSH). The acetylation reaction to form α-MSH is associated with the secretory process; its highest activity is present in the pituitary gland and certain brain regions.

The functional significance of N-terminal acetylation of $ACTH_{1-13}$ in the central nervous system is unknown although it may acutely regulate

$ACTH_{1-13}$ action. N-terminal acetylation of desacetyl-α-MSH to form α-MSH enhances some activities of $ACTH_{1-13}$ and virtually eliminates others. α-MSH injected daily to rats is 10 -100 fold more effective than desacetyl-α-MSH at increasing pigmentation, arousal, memory, attention, and excessive grooming. Desacetyl-α-MSH, however, is more effective than α-MSH at blocking opiate analgesia and opiate receptor binding *in vivo*. α-MSH and desacetyl-α-MSH also differentially affect feeding and weight gain. Weight gain of *agouti* obese mice is increased by subcutaneously administered desacetyl-α-MSH, as is food intake and fat pad weight, but α-MSH injections do not significantly increase food intake or body weight. Intracerebroventricular (ICV) α-MSH and $ACTH_{1-24}$ also inhibit food intake in food deprived and fed rats while ICV injections of desacetyl-α-MSH had no effect.

Despite these advances in our understanding of energy homeostasis, these efforts have not yielded clinically applicable parameters with which to predict or diagnose pathological imbalances that lead to obesity and/or type 2 diabetes. There is a need therefore for methods which would assist in the analysis and monitoring of energy metabolism, feeding and weight gain patterns.

It is an object of the present invention to ameliorate at least some of the disadvantages of the prior art methods, or at least provide useful alternatives.

SUMMARY OF THE INVENTION

Just as the measurement of "good" (HDL) and "bad" (LDL) cholesterol predicts cardiovascular risk, we have discovered that there is also "good" (α-MSH) and "bad" (desacetyl-α-MSH) melanocyte stimulating hormone (MSH) peptide, and the balance of these peptides predicts feeding/weight gain patterns, imbalances in energy homeostasis and obesity risk. This novel approach involves a new assay for quantitative risk assessment which will motivate people (by analogy with LDL/HDL ratios) to

change their lifestyle or undergo treatment to prevent the development of obesity.

The measurement of specific MSH peptides in human plasma and other biological fluids is possible following extraction and fractionation using high pressure liquid chromatography (HPLC), followed by classical RIA, according to modified methods described in the literature (Facchinetti, F., Bernasconi, S., Iughetti, L., Genazzani, A.D., Ghizzoni, L., Genazzani, A.R. Changes in dopaminergic control of circulating melanocyte-stimulating hormone-related peptides at puberty. Pediatric Research 38; 91-94, 1995; Mauri, A., Volpe, A., Martellotta, M.C., Barra, V., Piu, U., Angioni, G., Angioni, S., Argiolas, A. α-Melanocyte-stimulating hormone during human perinatal life. J Clin Endocrinol Metab 77: 113-117, 1993; Mauri, A., Martellotta, M.C., Melis, M.R., Caminiti, F., Serri, F., Fratta, W. Plasma alpha-melanocyte-stimulating hormone during the menstrual cycle in women. Hormone Research 34: 66-70, 1990)

Analysis of the abundance of and, particularly the ratios of, α-MSH and desacetyl-α-MSH in human circulation or other body fluid containing MSH peptides, are novel developments in the field of prediction and/or diagnosis of predisposition to obesity.

Accordingly, in one aspect, the present invention provides a method for assessing feeding and/or weight gain pattern in a mammal comprising the measurement of a melanocortin peptide in a sample taken from the mammal and comparison of the measured value with a reference value.

Accordingly, in another aspect, the present invention provides a method for predicting risk of obesity in a mammal comprising the measurement of a melanocortin peptide in a sample taken from the mammal and comparison of the measured value with a reference value.

Accordingly, in a further aspect, the present invention provides a method for diagnosing obesity in a mammal comprising the measurement of a melanocortin peptide in a sample taken from the mammal and comparison of the measured value with a reference value.

Accordingly, in yet another aspect, the present invention provides a method for diagnosing imbalance in energy homeostasis in a mammal comprising the measurement of a melanocortin peptide in a sample taken from the mammal and comparison of the measured value with a reference value.

Preferably, the melanocortin peptide measured is either α-MSH or desacetyl-α-MSH.

According to another aspect, the present invention provides a method for assessing feeding and/or weight gain pattern in a mammal comprising the measurement of at least two melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to a further aspect, the present invention provides a method for predicting risk of obesity in a mammal comprising the measurement of at least two melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to yet another aspect, the present invention provides a method for diagnosing obesity in a mammal comprising the measurement of at least two melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

According to yet another aspect, the present invention provides a method for diagnosing imbalance in energy homeostasis in a mammal comprising the measurement of at least two melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides and comparison of the value of the ratio with a reference value.

Preferably, the melanocortin peptide ratio taken is the ratio of desacetyl-α-MSH to α-MSH.

According to another aspect, the present invention provides a method for screening medicaments for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns, obesity or diabetes in a mammal to which the medicament has been administered comprising the measurement of a melanocortin peptide in a sample taken from the mammal, and comparison of the measured value with a reference value.

According to yet another aspect, the present invention provides a method for screening foods and/or diets for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns, obesity or diabetes in a mammal to which the medicament has been administered comprising the measurement of a melanocortin peptide in a sample taken from the mammal, and comparison of the measured value with a reference value.

Preferably, the melanocortin peptide measured is either α-MSH or desacetyl-α-MSH.

According to yet another aspect, the present invention provides a method for screening medicaments for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns, obesity or diabetes in a mammal to which the medicament has been administered comprising the measurement of at least 2 melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides, and comparison of the value of the ratio with a reference value.

According to a further aspect, the present invention provides a method for screening foods and/or diets for the adverse reactions of imbalance in energy homeostasis, feeding/weight gain patterns, obesity or diabetes in a mammal to which the medicament has been administered comprising the measurement of at least 2 melanocortin peptides in a sample taken from the mammal, the calculation of the ratios of the measured melanocortin peptides, and comparison of the value of the ratio with a reference value.

Preferably, the melanocortin peptide ratio taken is the ratio of desacetyl-α-MSH to α-MSH.

According to another aspect, the present invention provides a method of measuring the ratio of desacetyl-α-MSH to α-MSH comprising:

a. separating α-MSH and desacetyl-α-MSH in a sample,
b. measuring the amount of each of separated α-MSH and desacetyl-α-MSH originating in the sample, either directly or by subtraction of one of the amount of α-MSH or desacetyl-α-MSH from a measured amount of total MSH in a sample,
c. taking the ratio of the amounts of desacetyl-α-MSH to α-MSH.

Preferably, the separation procedure is selected from chromatography, electrophoresis, immunocapture, affinity capture including receptor-ligand capture or other affinity capture, and the like. It is also preferable that the quantitation procedure is selected from immunoassay including RIA, ELISA, Western blot, immunoprecipitation, and affinity capture, including receptor-ligand capture, peptide-nucleotide affinity capture or other affinity capture, and catalytic reaction-based assay, and the like. More preferably, the separation of the melanocortin peptide is by chromatography and the quantitation is performed by an immunoassay. Peptides may be isolated prior to quantitation. The preferred chromatographic method is HPLC and the preferred immunoassay is RIA. It is also preferable that the melanocortin peptides assayed accordingly are the peptides α-MSH and desacetyl-α-MSH.

It is also preferable that the sample be a biological fluid for example whole blood, plasma, serum, saliva, sweat, urine, amniotic fluid, cord blood, or cerebrospinal fluid or other body fluid.

According to a further aspect, the present invention provides a method of assessing risk of developing obesity and/or type 2 diabetes in a mammal comprising

a. separating α-MSH and desacetyl-α-MSH in a sample,
b. measuring the amount of each of separated α-MSH and desacetyl-α-MSH originating in the sample, either directly or by subtraction of one of the amount of α-MSH or desacetyl-α-MSH from a measured amount of total MSH in a sample,

c. taking the ratio of the amounts of desacetyl-α-MSH to α-MSH.

d. comparing the ratio of desacetyl-α-MSH to α-MSH with a reference ratio.

According to a further aspect, the present invention provides a method of diagnosing obesity in a mammal comprising

- separating α-MSH and desacetyl-α-MSH in a sample,
- measuring the amount of each of separated α-MSH and desacetyl-α-MSH originating in the sample, either directly or by subtraction of one of the amount of α-MSH or desacetyl-α-MSH from a measured amount of total MSH in a sample,
- taking the ratio of the amounts of desacetyl-α-MSH to α-MSH. comparing the ratio of desacetyl-α-MSH to α-MSH with a reference ratio.

BRIEF DESCRIPTION OF THE FIGURES.

Figure 1. Displacement of ^{125}I-α-MSH bound to rabbit antiserum (1:9000) by increasing amounts of melanocortin peptides. Insert: HPLC separation of α-MSH and desacetyl-α-MSH peptides.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is based on a surprising observation that a balance of MSH peptides in the circulation, in particular an increase in the desacetyl-α-MSH to α-MSH ratio, may correlate with, and be predictive of, the development of an imbalance in energy homeostasis, disturbance in feeding/weight gain patterns and ultimately obesity and diabetes. The absolute levels of individual MSH peptides may also serve this purpose. The absolute levels of combined MSH peptides may also serve this purpose.

Preferred embodiments of the invention will now be described by way of example only with reference to the following examples.

EXAMPLES

Example 1. Method for separation and detection/quantitation of α-MSH and desacetyl-α-MSH in plasma extracts

1.1. Extraction of Plasma using sep-Pak C18 Cartridge

Plasma (1-2 mL rodent or 10–20 mL human) was collect on ice and equal volume of 0.1M HCl add, and left for 30 minutes on ice. The plasma was spun for 30 minutes at 3300rpm at 4°C before use.

Sep Pak C18 cartridges (Waters Corporation, MA, USA) were pre-washed with 10mL methanol followed by 10 mL phosphate buffered saline (PBS). Sample was loaded onto column at flow rate of 5-10 mL per minute. 3mL of 10% methanol in 0.5M acetic acid was run over to elute non-specific or interfering substances (5-10 mL per minute). MSH peptides were eluted with 9mL 90% methanol in 0.5M acetic acid into silicanised tubes, then freeze dried to dryness with 900 μg polypep (Sigma-Aldrich, MO, USA) and 9 μL of 330 μM n-octyl-β-D-glucopyranoside (Sigma-Aldrich, MO, USA) added to each tube.

1.2 Separation of α-MSH and desacetyl-α-MSH using HPLC

Freeze dried mixture (after Sep-Pak extraction) was reconstituted in 150 μL HPLC buffer (acetonitrile: 0.1% trifluoroacetic acid (TFA) mixed at a ratio of 18:82). The sample was spun in Eppendorf tube to remove any precipitated material before transferring the sample to HPLC.

100μl of sample was injected onto HPLC C18 column (μ Bondpack, 39 x 300 mm, 10 μM size) and fractions collected by eluting with a linear gradient from 18-40% acetonitrile in 0.1% TFA at a flow rate of 1.5 mL/min. Fractions were collected into 6 mL siliconised glass kimble tubes each of which contained 15 μL of 10 mg/mL polypep and 1.5 μL of 330μM n-octyl-β-

D-glucopyranoside (Sigma-Aldrich, MO, USA). The fractions were freeze dried.

The retention times were: α-MSH, 8.6 minutes, and desacetyl-α-MSH, 6.5 minutes (Figure 1: Insert). It will be appreciated by those skilled in the art that this separation technique is applicable to samples other than plasma extracts. In fact it will be applicable without significant alterations to any biological fluid containing MSH peptides as well as samples of purified MSH peptides.

The separated α-MSH and desacetyl-α-MSH peptides are then quantitated using a sensitive and specific immunoreactive assay.

1.3 Radioimmunoassay of MSH peptides.

Freeze dried samples were reconstituted in RIA assay buffer (rodent – 200 μL; human – 300 μL). RIA assay buffer: 0.05 M phosphate buffer pH 7.4, 0.1 M NaCl, 0.5% BSA, 10 mM EDTA,

^{125}Iα -MSH was diluted to 10,000 cpm in RIA assay buffer.

α-MSH standards were prepared in RIA assay buffer: 0.00075, 0.001, 0.0015, 0.002, 0.003, 0.004, 0.005, 0.0075, 0.01, 0.015 ng/100μL

Desacetyl-α-MSH standards were prepared in RIA assay buffer: 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.5 ng/100μL

Assay procedure: tubes set up in duplicate with the following:

a) 100 μL standard or sample
b) 100 μL rabbit polyclonal antibody (KM4), 1:20,000 diluted in RIA assay buffer
c) Vortex and incubate overnight at 4°C
d) Add 100 μL ^{125}I- α -MSH (10,000 cpm) to each tube
e) Vortex and incubate overnight at 4°C
f) Prepare secondary antibody mix: 8% PEG 6000 in 0.01M PBS. 1% #2 sheep anti-rabbit gamma globulin, 0.025% normal rabbit serum.

g) Add 1mL secondary antibody mix to each tube
h) Vortex and incubate 1 hour at room temperature.
i) Spin at 3300rpm, 4°C for 45 minutes,
j) Drain off supernatant
k) Count residue in gamma counter

1.4 Development of polyclonal anti-α-MSH antibody

A high affinity antibody was raised following immunisation with synthetic α-MSH (N-Acetyl-SYSMEHFRWGKPV-NH_2) (purchased from Bachem, AG, Hauptstrasse 144, CH-4416, Bubendorf, Switzerland) conjugated to Keyhole limpet hemacyanin (KLH) according to conventional procedure described in well known literature (Antibodies. A Laboratory manual. E. Harlow & D. Lane. Cold Spring Harbor Laboratory, 1988) to each of 4 rabbits. A total of 8 injections were given at 3-week intervals. The details are as follows:

1. Four rabbits were immunised with 150 μg α-MSH conjugated to 300 μg KLH with glyceraldehyde per rabbit.
2. Immunisations were carried out by Animal Resource unit, University of Auckland. First immunisation used complete Freunds adjuvant. All other immunisations (3 weeks apart) used incomplete Freunds adjuvant.
3. One rabbit (KM4) developed antibodies that recognised both α-MSH and desacetyl-α-MSH.

1.5 Lactoperoxidase Iodination of α-MSH

1. Add 5 μL (2 μg) α-MSH in water to an Eppendorf tube.
2. Add 5 μl $Na^{125}I$ (0.5 μCi) to the α-MSH in Eppendorf tube.
3. Add 47 μL 0.1 M Na Acetate buffer, pH 5.6.
4. Add 10 μL lactoperoxidase (Sigma-Aldrich, MO, USA) freshly diluted in water (2 μg/100μL).
5. Add 5 μL H_2O_2 freshly diluted 1:7,500 in water.

6. Mix and incubate 5 minutes at room temperature.
7. Repeat steps 5 & 6 two more times.
8. Stop reaction by adding 500 µL PBS and 100 µL transfer buffer (Transfer buffer = RIA Assay buffer with 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) and 0.05% NaN_3.
9. Load mix onto a G2 chromatography column (Pharmacia K9) and elute with Transfer buffer.
10. Collect 1 mL fractions, count 10 µL of each fraction in gamma counter to identify the relevant protein peak.
11. Pool the 3-4 tubes on the descending side of the relevant protein peak.

To test the antisera 5 µg α-MSH was iodinated and purified. The iodinated material was incubated overnight at 4°C with diluted antiserum and increasing amounts of unlabeled melanocortin peptides. One rabbit developed a high affinity antibody which recognised both α-MSH and desacetyl-α-MSH and not ACTH, γ1, γ2, or γ3-MSH (Figure 1).

Example 2: Plasma MSH peptide content in normal and obese mice

Adult male mice were anaesthetised with halothane and decapitated. Blood was collected into ice cold tubes containing EDTA, The plasma was separated by centrifugation at 4000rpm for 10 minutes at 4°C. Plasma from 3-4 mice was pooled and mixed, extracted using Sep-Paks, and MSH peptides separated using HPLC and quantitated using RIA. Table 1 below shows the MSH data.

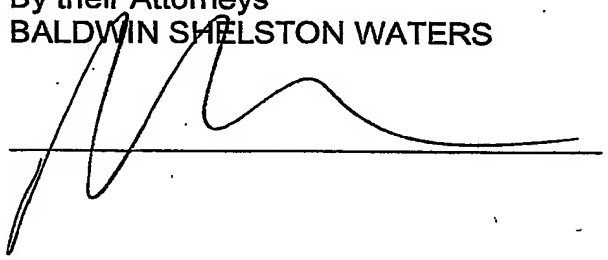
Table 1: Plasma from 3-4 mice were pooled and assayed for MSH peptides using HPLC and RIA assays.

MOUSE TYPE	α-MSH (pg/ml)	des-α-MSH (pg/ml)	α-MSH + des-α-MSH (pg/ml)	des-α-MSH/ α-MSH
A^{VY} yellow male (obese)	11.8	15.6	27.4	1.32
A^{VY} black male (lean)	19.5	16.4	35.9	0.84

The obese mice had a substantially higher des-α-MSH/α-MSH ratio than the lean mice. This was primarily due to a substantially lower level of α-MSH in the obese animals.

Although the invention has been described with reference to specific examples, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.

AUCKLAND UNISERVICES LIMITED
By their Attorneys
BALDWIN SHELSTON WATERS



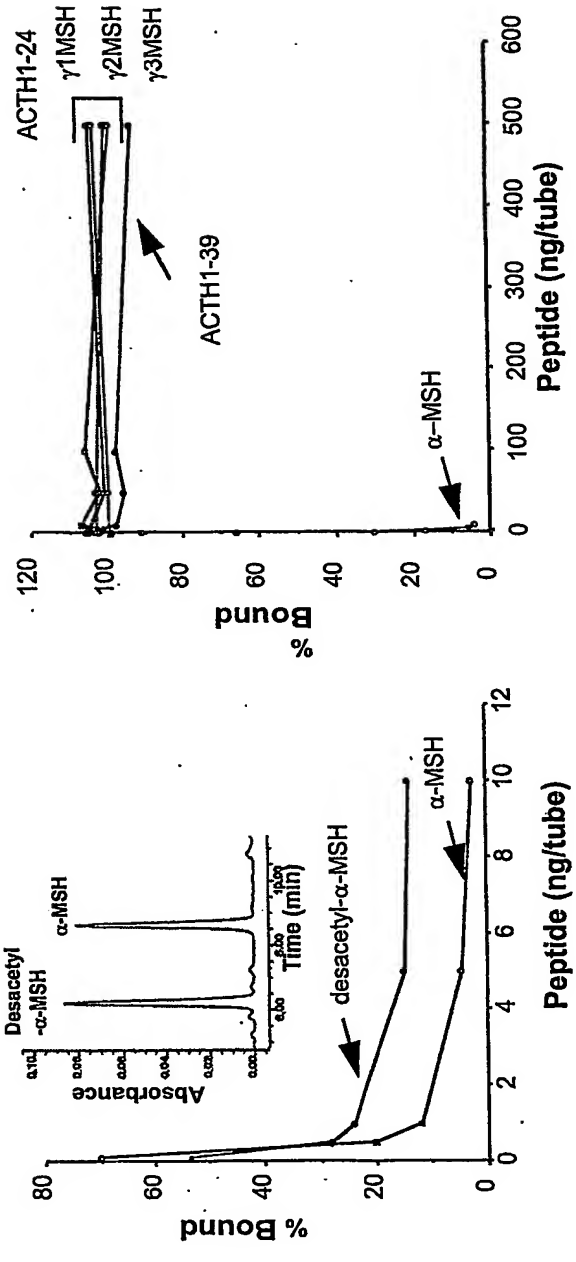

ACTH1-24
γ1MSH
γ2MSH
γ3MSH
ACTH1-39
α-MSH
Bound %
Peptide (ng/tube)
Absorbance
Time (min)
Desacetyl -α-MSH
α-MSH
desacetyl-α-MSH
α-MSH
% Bound
Peptide (ng/tube)

FIGURE 1